Staining of Lipids by the Periodic-Acid-Schiff Reaction. (18273)

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Schiff's reagent has afforded a means of visualizing aldehydic groups formed in tissue sections treated with oxidising reagents. In Bauer's procedure for polysaccharides(1) this reagent is used with chromic acid as the oxidant. McManus(2) and Hotchkiss(3) introduced oxidation by periodic acid for the same purpose, while Lillie(4) used successfully K permanganate. It has been widely assumed that the periodic-acid-Schiff staining method (PAS), as applied to tissue sections is specific for saccharidic compounds. Thus the PAS-positive granules in the liver have been described as glycogen granules, the presence of PAS-positive matter in capillary basement membranes and other sites has been considered as a proof that these structures consist of glyco-protein, and the positive staining of myelin sheaths with PAS has been ascribed to the presence of hexose-groups in the molecules of the myelin cerebrosides. Morrison and Hack(5) suggested that the PAS procedure could afford a means of differentiating Gaucher cells, containing the PAS-positive cerebrosides from Niemann-Pick cells. In a recent paper, McManus and Cason(6) adduced further evidence for their view that the PAS technic is specific for carbohydrates. They reported that the positive reaction of tissue structures with PAS can be inhibited by acetylation, but is restored by treatment of the acetylated sections with alkali. Experiments presented below indicate that the PAS method stains unsaturated lipids and sphingolipids as well as carbohydrates.

Experimental. Most experiments were performed using the technic of Coujard as described by Gomori(7). The compounds tested were mixed with plasma, and smeared on marked slides, about 10 compounds per slide. Human and rat livers and kidneys were examined in frozen sections after formalin fixation and in paraffin sections after Zenker's fixation. Specimens of livers of two cases of Niemann-Pick's disease* and spleens of 2 cases of Gaucher's disease were prepared from material which has been for 6 years or more in the laboratory either in the form of unstained paraffin sections, or as pieces of tissue preserved in formalin.

Results. (a) *Pure compounds.* Cerebroside†, sphingomyelin†, a lecithin-cephaline mixture†, saturated and unsaturated fatty substances obtained commercially: tristearin, stearic and palmitic acids; triolein,‡ oleic,‡ undecylenic, ricinoleic, nonenoic, crotonic, maleic, lauric and linoleic acids; and glycogen, failed to form a stained complex after 10 minutes treatment with Schiff's reagent.

After oxidation with periodic acid (according to Lillie's procedure)(1), with K permanganate 1% for 10 minutes, or with 5% chromic anhydride solution according to Bauer's technic, followed by a 10-minutes immersion in Schiff's reagent, the unsaturated lipids stained with the reagent, whereas the saturated lipids remained unstained. The

1. Lillie, R. D., Histopathologic Technic, Blakiston, Philadelphia, 1948.

2. McManus, J. F. A., *Nature,* 1946, v158, 202.

3. Hotchkiss, R. D., *Arch. Biochem.,* 1948, v16, 131.

4. Lillie, R. D., *Bull. Internat. Assn. Med. Mus.,* 1947, v27, 23.

5. Morrison, R. W. and Hack, M. H., *Am. J. Path.,* 1949, v25, 597.

6. McManus, J. F. A. and Cason, J. E., *J. Exp. Med.,* 1950, v91, 651.

7. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 354.

* One of them kindly supplied by Dr. Casper of the Beilinson Hospital, Petah Tiqvah.

† Kindly prepared from human brain by Dr. B. Shapira of the Department of Pathological Physiology.

‡ Batches of unsaturated lipids (triolein and oleic acid) which had been stored in the laboratory for a number of months in loosely stoppered bottles stained with the Schiff reagent.

intensity of staining varied, and was most marked in the sphingolipids. Oxidation in hydrogen peroxide of several concentrations gave irregular results, as most lipids came off the slides or turned into grayish substances when treated with this reagent. Positive staining of different intensities after H_2O_2 oxidation was observed with oleic acid, triolein, and the lecithin-cephalin mixture.

When the acetylation technic described by McManus and Cason was applied to smears of pure compounds, the materials were largely dissolved away. Small amounts of unsaturated lipid which remained on the slides after treatment with the pyridine-acetic anhydride reagent gave a positive PAS reaction. Experiments in which the attempt was made to remove the acetyl groups of the acetylated substances on Coujard slides by treatment with KOH 0.1 N according to McManus and Cason were inconclusive, as the smears did not adhere to the glass slides.

(b) *Histological material.* Acetylation abolished the staining ability of the liver cell cytoplasmic granules, and depressed the staining ability of the capillary basement membranes of the kidneys in human and rat paraffin sections. In this respect our findings are not in agreement with those reported by McManus and Cason. Blocks of rat liver and kidney were fixed rapidly in boiling 10% formalin, frozen sectioned and stained immediately afterwards, to avoid as far as possible oxidation of the unsaturated lipids by atmospheric oxygen. The acetylation procedure abolished the staining ability of practically all the liver cell granules, of most of the kidney cell granules, and reduced the intensity of staining of the tubular and capillary basement membranes and of the walls of vessels. After deacetylation of these sections by treatment with potassium hydroxide, the number of granules in the liver cells and kidney tubular cells was greatly reduced. Lipid granules in Niemann-Pick cells and in Gaucher cells stained positively with the PAS procedure. In paraffin sections of the Niemann-Pick cases, most lipid granules were dissolved by the solvents used in the preparation of the sections, but the remaining cytoplasmic substance contained PAS-positive material. The intensity of staining by PAS of the lipid granules in Gaucher and Niemann-Pick cells was reduced by the acetylation. The reduction was most marked in the material preserved in formalin. Treatment of acetylated Niemann-Pick and Gaucher sections with KOH resulted in the restoration of their stainability with PAS.

Discussion. The experiments reported above show that lipids containing double bonds stain with the PAS technic. The PAS reaction appears therefore to resemble in principle the oxidative plasmal reaction described by Verne(8), Gomori(9), Danielli (10) and Cain(11).

It is possible that the oxidation of unsaturated fatty acids by periodic acid follows the general rules of periodic acid oxidation [as formulated by Jackson(12)]. The first step in the oxidation of unsaturated fatty acids by mild oxidizing agents is considered to be the addition of two hydroxyl groups and resulting formation of a dihydroxy acid (Fittig)(13). In this stage, the fatty acid would come to correspond in its structure to the general requirements for periodic acid oxidation, and the molecule would be split with a formation of free aldehydic groups.

The valuable technic proposed by McManus and Cason in proof of the carbohydrate nature of the PAS-positive material of histological sections is seen to be inconclusive in certain circumstances:

1. When the tissue contains a PAS-positive lipid, which is soluble in acetic-anhydride-pyridine, as seems to have occurred in our

8. Cited by Lison, L., Histochimie Animale, Gauthier-Villars, Paris, 1936.

9. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 133.

10. Danielli, J. F., *Quart. J. Micr. Sci.,* 1949, v90, 67.

11. Cain, A. J., *Quart. J. Micr. Sci.,* 1949, v90, 75.

12. Jackson, E. L., in Organic Reactions, vol. II, edited by R. Adams, Wiley, New York, 1944.

13. Cited by Anschuetz, R. and Schroeter, G. in v.v.Richter's: *Chemie der Kohlenstoffverbindungen,* 11th ed. v.1, Cohen, Bonn, 1909.

frozen sections of liver cells and renal tubules lining cells.

2. When the tissue contains a PAS-positive lipid which is insoluble in acetic anhydride-pyridine, as appears to be the case in arterial walls and capillary basement membranes.

3. When the tissue contains sphingolipids.

The strongly positive PAS staining of sphingolipids can be attributed to the presence of the amino and hydroxyl groups on adjacent carbon atoms of the sphingosine moiety. In cerebrosides, the hexose group provides an oxidative point, while the double bond of the unsaturated fatty acid serves further to strengthen the reaction. The reduction by acetylation of the staining ability of Niemann-Pick cells preserved in formalin may be atributed to the oxidation of this material during storage. Dihydroxy groups probably appear at the double bond sites as a result of the partial oxidation by atmospheric oxygen, and together with the sphingosine 1 amino 2 hydroxy groups can then be acetylated and thus protected from attack by periodate.§

Conclusions. It must be concluded that the PAS method cannot be used to differentiate Niemann-Pick from Gaucher cells, and that it is not strictly specific for carbohydrates. A positive reaction may be given also by sphingolipids, and possibly by other substances having an amino group and a hydroxyl group on 2 adjacent carbons, as well as by unsaturated lipids.

Summary. 1. Unsaturated lipids stain with Schiff's reagent after oxidation with periodate (PAS reaction). The reaction is not inhibited by previous acetylation according to the technic of McManus and Cason. 2. Sphingolipids stain with the PAS technic whether they contained a carbohydrate moiety or not. Their reactivity may be ascribed to the presence of a hydroxy and an amino group on vicinal carbon atoms of the sphingosine moiety, and to the double bond in the fatty acid radicle. Acetylation reduces but does not abolish the staining ability of sphingolipids, since the double bond is not affected by the procedure. 3. The PAS method is not suitable for the differentiation of Niemann-Pick from Gaucher cells. 4. The ability of liver cell granules and of some renal tubule cell granules in fresh frozen sections to stain with PAS was abolished by acetylation, and largely restored by deacetylation. The fact that the staining of reticulin fibers in walls of vessels was not completely prevented by acetylation is considered as indicative of the presence of unsaturated fatty acid radicles in these structures.

§ The light staining of the lecithin-cephalin mixture may be ascribed both to the double bond of the fatty radical and to the serine moiety of cephalin.

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Arterial Volume Pulses and Peripheral Vascular Responses to Drugs. (18274)

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The work of Nyboer(1), Bloom, Kaufman, Nims and Nyboer(2), and others has shown that peripheral vasoconstriction produces a decrease and vasodilation an increase in the blood volume pulse of a body segment or organ. Certain difficult problems of instrumentation arise in recording simultaneous volume pulses from a number of total organs.

1. Nyboer, J., (in) *Medical Physics,* Vol. 1, O. Glasser (Ed.), The Year Book Publishers, Chicago, 1944, 340; Nyboer, J. (in) *Medical Physics,* Vol. II, O. Glasser (Ed.), The Year Book Publishers, Chicago, 1950, 736.

2. Bloom, W. L., Kaufman, S. S., Nims, L. F., and Nyboer, J., Natl. Res. Council, Com. on Aviation Med., Rep. No. 246, January 1944.